Intra-renal transfection of heat shock protein 90 alpha or beta (Hsp90α or Hsp90β) protects against ischemia/reperfusion injury

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ABSTRACT

Background. We previously reported that radicicol (Hsp90 inhibitor) induced a reduction in the renal blood flow and glomerular filtration rate, in part due to a reduction in urinary NO₂/NO₃ excretion, suggesting that Hsp90 regulates renal vascular tone in physiological conditions. However, there is a lack of information concerning Hsp90α or Hsp90β role on eNOS activity and their association with acute kidney injury (AKI) characterized by an inadequate NO production. This study evaluated the effects of Hsp90α or Hsp90β intra-renal transfection under ischemia/reperfusion (IR) injury.

Methods. Uninephrectomized (Nx) rats were intra-renally transfected through injections with Hsp90α or Hsp90β cloned into pcDNA3.1(+) or empty vector (EV) at 48 h before inducing IR, as indicated in the following groups: (i) Nx + sham, (ii) Nx + IR, (iii) Nx + IR + EV, (iv) Nx + IR + Hsp90α and (v) Nx + IR + Hsp90β. After 24 h, physiological, histopathological, biochemical and molecular studies were performed.

Results. IR-induced renal dysfunction, structural injury, tubular proliferation, the elevation of urinary Hsp72 and the reduction of urinary NO₂/NO₃ excretion. These alterations were associated with reduced eNOS–Hsp90 coupling and changes in the eNOS phosphorylation state mediated through a reduction in PKCα and increased Rho kinase expression. In contrast, intra-renal transfection of Hsp90α or Hsp90β prevented IR injury that was associated with the restoration of eNOS–Hsp90 coupling, eNOS activating phosphorylation and PKCα and Rho kinase levels.

Conclusions. Here we showed that eNOS–Hsp90 uncoupling plays a critical role in promoting NO reduction during IR. This effect was effectively reversed through Hsp90α or Hsp90β intra-renal transfection, suggesting their implication in regulating NO/eNOS pathway and the renal vascular tone.

Keywords: acute kidney injury, nitric oxide, renal blood flow, renal dysfunction, transfection

INTRODUCTION

Renal ischemia/reperfusion (IR) is one of the major causes of acute kidney injury (AKI) among hospitalized patients. The hallmark of ischemic AKI is the reduction in the renal blood flow (RBF) [1], damaging the endothelial and proximal tubular cells [2–5]. The relevance of inadequate nitric oxide (NO) synthesis during AKI has been highlighted in several studies [6, 7] (for review see ref. [3]). It has been demonstrated that endothelial nitric oxide synthase (eNOS) expression is reduced early in endothelial cells after ischemia through the recruitment of histone de-acetylases to the promoter region of the eNOS gene, inhibiting its transcription and reducing NO synthesis [6]. Accordingly, it has been shown that Brown Norway rats are more resistant to myocardium IR injury than Dahl S rats because Brown Norway rats exhibit higher Hsp90-eNOS interaction, tetra-hydrobiopterin bioavailability and NO synthesis than observed in Dahl S rats [8, 9]. In addition, ischemic pre-conditioning protects against IR injury through enhanced Akt-mediated eNOS phosphorylation at Ser1177, which is abolished through the inhibition of eNOS before ischemic pre-conditioning [10, 11]. Taken together, these studies suggest that mechanisms that re-establish NO production would be beneficial for the treatment of AKI.
The 90-kDa heat shock protein subfamily (Hsp90) is a group of highly conserved stress proteins ubiquitously expressed. These proteins are molecular chaperones that facilitate protein folding, regulate quality control, modulate protein activities, guide proteins to specific cellular locations and so forth. Five Hsp90 isoforms have been described, which differ in cellular localization and abundance [12]. In particular, Hsp90α and Hsp90β are the major cytosolic isoforms and are two of the most abundant proteins in the cell, sharing ~85% sequence identity at the protein level. Hsp90 interacts with and modulates the activity of more than 300 proteins (http://www.picard.ch/downloads/Hsp90interactors.pdf), including multiple transcription factors, enzymes and kinases; thus, these proteins regulate many cellular processes [13–15].

eNOS is a major source of NO in the endothelium. Hsp90-eNOS coupling promotes NO production [16]. In contrast, when this interaction is disrupted, NO generation is reduced and eNOS is converted into a superoxide anion producer [17]. In addition, the protein kinase C βII (PKCβII) or Rho Kinase (RhoK)-mediated phosphorylation at threonine 495 (Thr495) inactivates eNOS [18, 19], as this phosphorylation prevents eNOS association with calmodulin [20, 21]. In response to an appropriate stimulus, eNOS dissociates from caveolin-1 (Cav1), facilitating the interaction with Hsp90. This interaction prompts a conformational modification in eNOS, which exposes serine 1177 (Ser1177) to protein kinase Cα (PKC-α) or protein kinase B (Akt)-mediated phosphorylation, leading to eNOS dimerization and enhanced NO generation [21, 22–24].

In a previous study, we showed that Hsp90α and Hsp90β are abundantly expressed along the nephron [25] and that inhibition of Hsp90 with radicicol reduces RBF and the glomerular filtration rate (GFR). These effects have been associated with a reduction in NO synthesis and increased eNOS phosphorylation at Thr495 [26], suggesting that Hsp90 is a regulator of renal vascular tone through modulating eNOS activity in the kidney.

Because AKI is associated with inadequate NO production and information concerning the specific role of Hsp90α or Hsp90β on eNOS and renal function is lacking, the aim of this study was to evaluate the effects of intra-renal Hsp90α or Hsp90β transfection under ischemic conditions.

**Materials and Methods**

**Plasmid constructs**

Hsp90α and Hsp90β genes were PCR amplified using cDNA synthesized from total rat RNA, as previously described [27]. The amplified fragments of 2260 bp for Hsp90α and 2220 bp for Hsp90β were cloned into the pcDNA3.1 (+) vector (Invitrogen) using the Not I and Xba I restriction sites. The identity of the constructs was confirmed through automatic sequencing.

**Hsp90α or Hsp90β plasmid isolation**

The plasmids containing the Hsp90α or Hsp90β sequences (pc-Hsp90α or pc-Hsp90β, respectively) were isolated using the general strategy for large-scale isolation according to Qiagen Science (MD). The concentration and purity of the DNA were assessed through 1% agarose electrophoresis using molecular weight marker standards (λ DNA/Hind III fragments from Invitrogen, Carlsbad, CA), and the results were consistent with the molecular weights reported for the Hsp90α and Hsp90β genes. The DNA plasmid yield was 50–80 μg in a 50-ml starting culture.

**Experimental protocols**

All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and approved through the Animal Care and Use Committee of our Institution.

In the first set of experiments, we investigated the role of intra-renal transfection of Hsp90α or Hsp90β under physiological conditions. For this purpose, we included four groups: (i) sham operated (S), (ii) transfected with the empty vector (EV), (iii) transfected with pcDNA3.1/Hsp90α and (iv) transfected with pcDNA3.1/Hsp90β. Each group contained five rats.

In the second set of experiments, we studied the effect of the intra-renal transfection of Hsp90α or Hsp90β on IR injury in five groups of uninephrectomized rats (Nx): (i) Nx + sham surgery (n = 6), (ii) Nx + IR (n = 7), (iii) Nx + IR + intra-renal transfection with EV (n = 7), (iv) Nx + IR + intra-renal transfection with Hsp90α (n = 7) and (v) Nx + IR + intra-renal transfection with Hsp90β (n = 8). All animals were studied at 24 h after inducing ischemia and maintained in a 12 h–night cycle and with free access to water and food.

**Intra-renal transfection of Hsp90α or Hsp90β**

Male Wistar rats weighing 270–300 g (12–15 weeks) were used in this study. The rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad with a rectal probe to maintain a core body temperature at 37°C. The right kidney was removed immediately prior to transfection in all the rats included in protocol 2. The left renal artery was perfectly dissected. The complexes of liposomes and the corresponding vectors were prepared as follows: 100 μL of Lipofectamine 2000 (Invitrogen) + 50 μg of the vector + physiological solution in a final volume of 200 μL. Subsequently, a non-traumatic clamp was placed over the left renal artery and another clamp was placed over the left renal vein. The complexes were injected into the renal artery in the space between the clamps and the kidney using a 31-mm/31-G insulin needle. After 2 min, the clamps were released. The incision was closed in two layers with 3–0 sutures, and the rats were allowed to recover from the surgery. In addition, three rats were transfected with the pcDNA 3.1 containing the sequence for green fluorescent protein (GFP).

**Ischemia/reperfusion model**

Forty-eight hours after nephrectomy and transfection, the rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad to maintain core body temperature at 37°C. The left renal...
pedicle was isolated, and left renal ischemia was induced through the collocation of a non-traumatic clamp in each renal artery for 30 min. Ischemia was visually verified through changes in kidney color. Reperfusion was achieved after releasing the clips and confirmed through the return of oxygenated blood to the kidneys. The incision was closed in two layers with 3-0 sutures and reperfusion was maintained for 24 h before sacrifice. In the sham groups, the surgery under anesthesia, laparotomy and renal pedicle dissection were performed without clamp collocation.

Evaluation of functional parameters
When the rats recovered from surgery, they were housed in metabolic cages, urine was collected for 24 h and stored at −80°C. Once urine was collected, the rats were anesthetized with sodium pentobarbital (30 mg/kg) and placed on a homeothermic table. The femoral arteries were catheterized using polyethylene tubing (PE-50). The mean arterial pressure (MAP) was monitored using a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA) for all experiments. An ultrasonic transit-time flow probe was placed around the left artery and filled with ultrasonic coupling gel (HR Lubricating Jelly, Carter-Wallace, New York, NY) to record the renal blood flow for 10 min. Blood samples were collected at the end of the study. The serum creatinine concentrations were measured using a Quantichrom creatinine assay kit (DICT-500). Urinary protein excretion was measured using the TCA turbidimetric method [28].

Histopathological studies
At the end of the experiment, half of the remnant kidney was removed and immediately frozen for molecular studies. The other half was fixed in a freshly prepared 4% formalin buffer. After appropriate dehydration, the kidney slices were embedded in paraffin, sliced into 4-μm sections and stained via periodic acid-Schiff (PAS). Ten subcortical fields were randomly recorded from each rat slide using a Nikon light microscope equipped with a digital camera. In each microphotograph, the tubular affected area was blindly analyzed. Tubular damage was characterized as the loss of brush border, lumen dilatation, and detachment from the basement membrane. The percentage of the affected tubular area was assessed through morphometric analysis using Eclipse net software (magnification 400×). The damaged tubular area was expressed as a proportion of the affected tubular area and total tubular area.

GFP immunofluorescence
In the rats transfected with GFP, the left kidney, perfused with PBS, was harvested and a slice of the kidney was frozen and sectioned into 5-μm thick slices. For GFP staining, cryosections from three rats transfected with EV and three rats transfected with GFP were fixed in 10% acetone for 5 min, and permeabilized with 0.1% Triton-X for 5 min. After washing, the sections were incubated for 40 min with Alexa Fluor 647 anti-GFP antibody (Biolegend, 1:300). The images were captured on a Zeiss LSM 5 Pascal confocal microscope (Göttingen, GE).

Ki-67 immunohistochemistry
Four-micrometer sections from paraffin embedded tissues were de-waxed and probed against Ki-67 (Abcam antibody ab66155). Twenty cortical fields (magnification 400×) were randomly recorded from each kidney slide using a digital camera incorporated in a Nikon light microscope. The nuclei of tubular cells that were positive for Ki-67 were counted and reported as Ki-67-positive cells per field.

Western blot analysis
Total proteins were isolated from two different kidney pools obtained from three different rats of each group and homogenized in lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40) containing complete protease inhibitor (Roche). The protein samples containing 50 μg of total protein were resolved through 8.5% SDS-PAGE electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Millipore), except for the PKCα analysis, in which 5 μg of total protein was loaded. The upper membrane sections were blocked with 5% blottoing-grade non-fat dry milk, and subsequently incubated in 0.1% blotting-grade non-fat dry milk containing the appropriate antibodies. Antibodies against Hsp90α (Abcam; 1:5000), Hsp90β (Abcam; 1:5000), eNOS (BD Transduction Laboratories; 1:5000), P-Ser1177-eNOS (Cell Signaling; 1:500), P-Thr495-eNOS (Cell Signaling; 1:500), PKC-α (Santa Cruz; 1:20000), PKC-β (Santa Cruz; 1:100000), Akt (Invitrogen; 1:5000), P-Ser473-Akt (Invitrogen; 1:500), FoxO3 (Santa Cruz; 1:5000) and P-FoxO3 (Cell Signaling; 1:1000) were used. After incubation with primary antibody, the membranes were washed and incubated with the appropriate secondary antibodies (anti-mouse, anti-goat or anti-rabbit IgG-HRP). As a loading control, lower membrane sections were incubated with goat anti-β-actin antibody (1:5000 dilution; Santa Cruz Biotechnology, Santa Barbara, CA) overnight at 4°C. β-actin detection was performed using donkey anti-goat IgG-HRP (1:5000; Santa Cruz Biotechnology). The proteins were detected using an enhanced chemiluminescence kit (Millipore) and autoradiography, according to the manufacturer’s recommendations. The bands were scanned for densitometric analysis and the results were analyzed.

Analysis of Hsp90 dimer and monomer conformation
The Hsp90α or Hsp90β dimer/monomer ratio was evaluated through western blotting for non-denatured proteins. Non-boiled samples, containing 10 μg of total protein, were resolved through 7.5% PAGE at 4°C. The proteins were transferred onto a polyvinylidene difluoride membrane, and the western blot analysis for Hsp90α or Hsp90β was performed as described above.

Immunoprecipitation analysis
eNOS protein was immunoprecipitated using a protein G immunoprecipitation (IP) kit (Sigma Aldrich, Inc., St Louis, MO). For this purpose, eNOS was immunoprecipitated from 1 mg of pooled protein from each group using 5 μg of anti-eNOS antibody (BD Transduction Laboratories). The immunoprecipitated proteins were eluted through boiling in

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Laemmli sample buffer, separated through 8.5% SDS-PAGE, transferred onto PVDF membranes and subjected to western blotting. The membrane was blocked with 5% non-fat milk in TBS–Tween (0.1%), and subsequently the upper membrane section was immunoblotted for eNOS, and the lower membrane section was immunoblotted for Hsp90α. The same procedure was performed for Hsp90β.

**Urinary Hsp72 levels**

The urinary Hsp72 levels were analyzed using a commercially available high-sensitivity ELISA kit (Enzo Life Sciences EKS-715, MI, USA) according to the manufacturer’s instructions. Briefly, the samples and standards were added to wells coated with a mouse monoclonal antibody. Hsp72 was captured by the antibody and then detected after adding an Hsp72 rabbit polyclonal detection antibody. Both antibodies were specific for inducible Hsp72 and did not react with other members of the HSP70 family. A horseradish peroxidase conjugate bound to the detection antibody and color development was accomplished after the addition of the tetramethylbenzidine substrate, and the reaction was terminated with an acid stop solution. The optical density of the samples was read at 450 nm using a plate reader, and the results compared with the standard curve generated from known concentrations of recombinant Hsp72.

**Urinary nitrates and nitrates excretion**

NO production was indirectly determined in the urine samples from each rat using the colorimetric Nitric Oxide Assay Kit (Oxford Biomedical Research, Inc., Oxford, MI). In this assay, the sample nitrates are reduced to nitrites through nitrate reductase, followed by nitrite quantification using Griess reagent and detection at 540 nm.

**Statistical analysis**

The results are presented as means ± SE. The significance of the differences among groups was tested through ANOVA using Bonferroni’s correction for multiple comparisons. All comparisons passed the normality test. The statistical significance was defined as P < 0.05. Sigma Plot 10.0 was used for the graphical representation of the data.

**RESULTS**

**Intra-renal transfection of Hsp90α or Hsp90β**

Rat Hsp90α or Hsp90β cDNA was cloned onto the mammalian expression vector pcDNA3.1, packed into liposomes and subsequently transfected into the left renal artery of normal rats. First, we established the optimal period in which the maximal over-expression of Hsp90α or Hsp90β was reached. Subsequently, the animals were examined at 24, 48 and 72 h after intra-renal transfection to obtain the temporal expression of Hsp90α or Hsp90β levels in the kidney. As shown in Figure 1A and B, the intra-renal transfection of each plasmid induced a significant increase in the protein levels of Hsp90α or Hsp90β. In both cases, maximal over-expression was detected at 48 h post-transfection. Thus, all subsequent experiments were performed at 48 h post-transfection.

The intra-renal transfection of the same vector containing GFP revealed that transfection occurred in both the vascular and tubular epithelium, as depicted in Figure 1D and E.

**Effects of intra-renal transfection of Hsp90α or Hsp90β in normal renal hemodynamic conditions**

Once we established the optimal conditions for the intra-renal transfection of Hsp90α or Hsp90β in the kidney, we assessed whether Hsp90α or Hsp90β transfection affected the MAP or renal hemodynamics in normal rats. As shown in Figure 2, the MAP, RBF and serum creatinine or proteinuria levels remained unchanged in rats transfected with either Hsp90 isoform. These findings showed that the renal artery and renal hemodynamics were not damaged or altered after the injection of the genomic material.

**Effects of the intra-renal transfection of Hsp90α or Hsp90β in an ischemic/reperfusion setting**

The left kidney of uninephrectomized rats was transfected with Hsp90α or Hsp90β at 48 h before renal ischemia was performed. All animals were studied at 24 h after reperfusion. As is shown in Figure 3A, the MAP was not modified as a result of renal ischemia or transfection with empty, Hsp90α or Hsp90β vectors. As expected, IR injury was characterized by a reduction in RBF (Figure 3B) and a significant increase in serum creatinine (Figure 3C) and urinary protein excretion (Figure 3D). Interestingly, the transfection of either Hsp90α or Hsp90β prior to renal ischemia protected against IR injury, as reflected by the prevention of renal hypoperfusion, the elevation of serum creatinine and abnormal proteinuria. Hsp90α or Hsp90β-induced renoprotection was also documented through histopathological observations. As expected, renal IR caused severe tubular damage and a similar effect was observed in IR rats transfected with EV (Figure 4A and B, respectively). In contrast, Hsp90α or Hsp90β transfection significantly reduced tubular injury, as shown in Figure 4C and D. The morphometric analysis showed that the percentage of tubular injured area was significantly reduced from 40.2 in untranslated rats to 20.0 and 19.6% in Hsp90α or Hsp90β transfected animals, respectively (Figure 4E). In addition, the renal protection conferred by Hsp90α or Hsp90β was also confirmed after quantifying the urinary levels of Hsp72 as a sensitive marker of tubular damage induced through renal ischemia, as previously reported [29] (Figure 4F).

Renal tubular cell proliferation was assessed by immunohistochemistry against Ki-67, a nuclear protein marker of all active cell cycle phases (G1, S, G2 and mitosis) [30]. Representative microphotographs of Ki-67 immunohistochemistry are shown in Figure 5. Ischemia/reperfusion injury was associated with a significant increase in tubular cell proliferation (Figure 5B and G) compared with sham-operated rats (Figure 5A and F), 11.0 ± 1.7 versus 2.9 ± 0.2, tubular positive cells per field (P < 0.05). The extensive tubular proliferation observed in IR groups was not modified by Hsp90α, 12.4 ± 3.5 (Figures 5D and I) or Hsp90β 14.3.0 ± 1.3 (Figure 5E and J). Although the tubular proliferation was similar, it is
noteworthy the preservation of tubular architecture in these Hsp90 transfected kidneys.

Studies have previously demonstrated that IR injury is associated with a reduction in NO generation [6, 7, 31, 32]. Therefore, we evaluated whether the improvement of RBF in IR groups transfected with Hsp90α or Hsp90β resulted from the re-establishment of NO generation. Indeed, the intra-renal transfection of Hsp90α or Hsp90β was associated with the preservation of urinary NO2/NO3 excretion (Figure 6A). This effect did not reflect the up-regulation of eNOS expression,
but was accompanied by changes in the eNOS phosphorylation (Figure 6B and C). The reduction of NO in animals under IR, without or with EV transfection, was associated with an increase in eNOS phosphorylation at Thr495 and reduction of eNOS phosphorylation at Ser1177, which inactivates and activates eNOS, respectively [26, 33]. The transfection of Hsp90α or Hsp90β prevented both, the increase in the inactivating eNOS phosphorylation and the reduction in the activating eNOS phosphorylation (Figure 6B and C).

To understand the mechanisms underlying the intra-renal transfection of Hsp90 isoforms and the resulting changes in eNOS phosphorylation, we evaluated whether the interactions between each Hsp90 isoform with eNOS were altered. Compared with control rats not exposed to ischemia, the interaction between eNOS and Hsp90α was significantly reduced after IR. Whereas, the Hsp90α transfection prior to IR enhanced eNOS–Hsp90α interactions (Figure 7A). Similarly, this interaction was enhanced when Hsp90β was transfected prior to IR (Figure 7B). Because it has been demonstrated that the dimeric form of Hsp90 is more effective for binding and/or activating target proteins [14, 34], we assessed the dimer/monomer ratio of Hsp90α or Hsp90β among the studied groups. No differences in dimer/monomer ratio of these proteins were observed among the groups (Figure 7C and D).

PKCα and Akt/PKB are the kinases responsible for eNOS phosphorylation at Ser1177 [21, 35]. Consistent with the reduction in eNOS-activating phosphorylation in IR groups without or with EV transfection, the PKCα protein levels were significantly reduced and they were restored through transfection with Hsp90α or Hsp90β (Figure 8A). Although, reduced PKCα-mediated phosphorylation at Thr638 was observed, no differences were detected among the groups when the P-Thr638-PKCα/PKCα ratio was analyzed (Figure 8B). We observed that instead of being reduced, Akt/PKB was significantly activated during IR injury. Thus, Akt-mediated phosphorylation at Ser473 was augmented in these animals, as shown in Figure 8C. Because Akt/PKB activity has been associated with promoting cell proliferation under ischemic conditions [36, 37], we also evaluated the expression and activity of the forkhead protein FKHRL1, also known as FOXO3A, as a transcription factor for cellular proliferation [38]. As shown in Figure 8D, FOXO3A phosphorylation at Thr32 was significantly increased in the IR transfected group with the EV, and this effect was not observed in the IR groups with either Hsp90α or Hsp90β intra-renal transfection.

Furthermore, we evaluated the major kinases responsible for inactivating eNOS phosphorylation. Specifically, we studied the renal expression of PKCβII and RhoK as shown in Figure 9. The expression of neither PKCβII nor its active phosphorylated form (at Ser660) was different among the studied groups (Figure 9A). In contrast, the RhoK expression was significantly enhanced in the IR group without or with EV transfection, an effect prevented through the intra-renal transfection of Hsp90α or Hsp90β (Figure 9B). Thus, these results were consistent with the eNOS phosphorylation at Thr495.

**DISCUSSION**

In this study, we showed that the intra-renal transfection of Hsp90α or Hsp90β protects against renal damage induced through IR. The protective effect was associated with the restoration of eNOS–Hsp90 coupling, reestablishment of PKCα
FIGURE 4: Effect of Hsp90α or Hsp90β intra-renal transfection on tubular injury induced through IR. Representative images of PAS-stained sections from (A) Nx + IR, (B) Nx + EV + IR, (C) Nx + Hsp90α + IR and (D) Nx + Hsp90β + IR groups (n = 6). Original magnification: 400×. (E) Morphometric analysis of the percentage of the injured tubular area. (F) Urinary Hsp72 served as a biomarker for tubular injury. *P < 0.05 versus the Nx group and ¥P < 0.05 versus Nx + IR and Nx + EV + IR groups.

FIGURE 5: Effect of Hsp90α or Hsp90β intra-renal transfection on tubular cell proliferation. Representative images of Ki-67 immunohistochemistry from (A and F) Nx, (B and G) Nx + IR, (C and H) Nx + EV + IR, (D and I) Nx + Hsp90α + IR and (E and J) Nx + Hsp90β + IR groups. Original magnification: 100× (A–E) and 400× (F–J). The number of Ki-67 positive cells per field is stated below the microphotographs. *P < 0.05 versus the Nx group.
levels and reduction of Rho kinase expression, effects that were able to return eNOS phosphorylation to its basal state. These events not only restored NO levels but also prevented the reduced renal blood flow and extension of AKI induced through ischemia.

Rats subjected to IR and transfected with either Hsp90α or Hsp90β showed no reduction in RBF and abnormal proteinuria, as observed in IR un-transfected rats or the groups transfected with the EV. Although, there was no difference in serum creatinine levels among the groups using ANOVA, it is clear that rats transfected with Hsp90α or Hsp90β exhibited better renal function. Moreover, further characterization of the renoprotective effect using light microscopy and morphometric analysis showed an important preservation of the tubular epithelium. The functional and structural protection conferred by Hsp90α or Hsp90β transfection suggests that these proteins were able to maintain a better endothelial and epithelial cellular homeostasis. In addition, transfection of Hsp90α or Hsp90β was only observed in the transfected kidney, and not in other tissues such as liver, intestine, heart, or the contralateral kidney (data not shown).

Moreover, when we evaluated Ki-67 staining, the expected tubular proliferation after 24 h of reperfusion was not modified by Hsp90α or Hsp90β transfection. We cannot exclude, however, that changes in the proliferation rate may occur at earlier or later stages of reperfusion, since the tubular epithelium was better preserved in Hsp90α transfected rats.

Recently, we showed that the levels of urinary Hsp72 are an early and sensitive biomarker of AKI induced through an ischemic episode [29]. Indeed, we observed that IR groups un-transfected or transfected with the EV exhibited elevated urinary Hsp72 excretion, reflecting the degree of tubular
damage. Consistently with the renal functional and structural analyses, rats transfected with Hsp90α or Hsp90β before IR exhibited urinary Hsp72 levels similar to those in the uninephrectomized control group, reflecting slight renal injury in these animals. Thus, these findings show that the intra-renal transfection of Hsp90α or Hsp90β protected the animals from AKI. Similar results were obtained in another model of myocardial ischemic injury with Hsp90 transfection [39]. Paradoxically, it has been observed that administration of Hsp90 inhibitors such as, geldanamicyn, 17AAG and 17DMAG also protects against renal injury induced by ischemia. This apparent discrepancy could be explained by the fact that prolonged use of these inhibitors actually induces an up-regulation of Hsp70 and Hsp27 in the kidney, although Hsp90 was also induced in renal adenocarcinoma cells. These inhibitors induced these events through promoting heat shock factor-1 trimerization, nuclear translocation and phosphorylation [40, 41]. The exact mechanism by which Hsp70 over-expression conferred renoprotection against an ischemic insult remains poorly understood, but it has been proposed that Hsp70 reduces inflammatory Hsp90 client proteins, (ii) inducing IκB stabilization and/or (iii) preventing NFκB p65 translocation (for review see [42]).

A reduction in the RBF is critical for the initiation and extension of AKI. One of the pathophysiological events that occurred during AKI is the affection of vascular tone mediated, in part, by the reduction of NO derived from eNOS and endothelial damage [6, 7] (for review [3]). In this study, we confirmed that under ischemic conditions, rats exhibited a significant decrease in urinary NO metabolites, confirming that NO reduction contributes to IR pathophysiology. Consistently, several studies have shown that mechanisms that increase eNOS activity protect against IR injury [11, 43–45, 46, 39].

At the basal level, eNOS is primarily localized to caveolae, interacting with Cav1; this interaction maintains eNOS in the inactive state. After endothelial stimulation, followed by an increase in shear stress or cytoplasmic calcium, calmodulin is activated, which in turn weakens eNOS-Cav1 interactions and promotes eNOS activation through association with Hsp90. However, little is known about the specific role of Hsp90 cytosolic isoforms, Hsp90α and Hsp90β, in both renal...
physiological and pathophysiological conditions. In previous studies, we demonstrated that Hsp90 is involved in the maintenance of kidney function, as the acute inhibition of Hsp90 with radicicol-induced renal vasoconstriction, an effect associated with NO reduction and the subsequent decrease in the glomerular filtration rate [26]. In addition, we have also observed that Hsp90α transfection in human kidney epithelial cells (HEK-293) in culture displayed a significant increase in NO generation mediated through an increase in the Akt-mediated phosphorylation of eNOS at Ser1177Akt, whereas Hsp90β HEK-transfected cells exhibited a reduction in NO generation. Since AKI is characterized by NO reduction, we assumed that intra-renal transfection of Hsp90α may improve renal injury induced by IR, whereas Hsp90β transfection may worsen it. However, in the present study, we observed that the intra-renal transfection of both Hsp90α and Hsp90β protected the kidney against IR injury through a mechanism that included NO restoration. It is possible that this discrepancy reflects the different studied systems, i.e. in vitro versus in vivo. Another possibility is that the restoration of renal blood flow and NO in Hsp90α- or Hsp90β-transfected kidneys, restored eNOS–Hsp90 coupling in endothelial cells, as these isoforms have similar effects on the NO/eNOS pathway, which differ from those observed in epithelial cells in vitro.

Since Hsp90 regulates multiple cellular processes, the renoprotection conferred by Hsp90α or Hsp90β in ischemic animals may result from the activation of several pathways, being one of them the NO pathway. Therefore, to determine the mechanisms underlying the restoration of NO in the IR groups transfected with either Hsp90α or Hsp90β, we evaluated the phosphorylation state of two major residues implicated in eNOS activity. Rats subjected to IR exhibited a significant reduction in the eNOS-activating phosphorylation at Ser1177 together with a significant increase in eNOS-inactivating phosphorylation at Thr495. Interestingly, transfection with either Hsp90α or Hsp90β restored the changes in the eNOS phosphorylation state to a similar extent as that observed in the uninephrectomized control group. Moreover, our co-IP analysis showed that both isoforms interact with eNOS, but eNOS–Hsp90α interaction is reduced in kidneys of un-transfected and EV-transfected IR rats. In contrast, rats transfected with Hsp90α or Hsp90β exhibited increased interactions with eNOS. Previous studies have shown that eNOS–Hsp90 coupling promotes eNOS-activating phosphorylation [33], whereas eNOS-inactivating phosphorylation at Thr495 resulted from eNOS–Hsp90 uncoupling [26]. Thus, it is possible that during IR, Hsp90 mainly is re-establishing cellular homeostasis, and the eNOS–Hsp90 interaction is reduced with the concomitant decrease in NO synthesis as was observed by IP analysis. Another possibility is that Hsp90 does not work efficiently during ischemic conditions. Indeed, a previous study showed that Hsp90α and Hsp90β in the presence of peroxinitrites as occurs in IR injury [47, 48] suffers from tyrosine nitrosylation that impedes Hsp90 chaperone activity [49].

The dimeric form of Hsp90 is required for the activation and stabilization of its target proteins, a process dependent on ATP hydrolysis [50–52]. In humans and mice, Hsp90β is less frequently observed as a dimer than Hsp90α, this difference is mediated through the C-terminal dimerization domain, in which two residues impede Hsp90β dimerization [53, 54]. Similarly, in the present study we showed that Hsp90α was primarily observed as a dimer and Hsp90β was observed as a monomer in the rat kidney. However, the intra-renal transfection of either Hsp90α or Hsp90β restored eNOS activity in the IR groups, suggesting that Hsp90β may function as a monomer or heterodimer or in cooperation with other co-chaperones. Indeed, Chadli et al. [55] demonstrated that Hsp90β preferentially associates with general cell UNC45 (GCUNC45), forming a co-chaperone complex.

It has been shown that eNOS activation through Hsp90 is mediated, in part, by a scaffolding effect that enhances the

**Figure 9:** Expression of PKCβII and Rho kinases, which potentially phosphorylate eNOS at Thr495. (A) The protein levels of P-Ser660 PKC-βII and total PKC-βII. The upper insets show the representative autoradiographs from the western blot analysis and the lower graph the densitometric analysis. (B) Rho K protein levels. The upper insets show the representative autoradiographs from the western blot analysis and the lower graph represents the densitometric analysis *P < 0.05 versus the Nx group.
interaction between eNOS and the active serine/threonine kinase Akt/PKB [21], thereby promoting eNOS phosphorylation at Ser1177. Akt/PKB activity is also regulated through Hsp90, preventing PP2A-mediated Akt dephosphorylation [35]. In addition, PKCα might also phosphorylate the serine residue of eNOS [22]. In the present study, we demonstrated that the IR group showed lower PKCα expression, potentially reflecting the reduced phosphorylation of eNOS at Ser-1177. The overexpression of Hsp90α or Hsp90β restored the levels of this kinase, which could explain the re-establishment of eNOS phosphorylation at Ser-1177. These findings are also consistent with previous studies showing that Hsp90 is involved in maintaining the stability of PKCα [56]. We also observed that un-transfected and EV-transfected IR rats exhibited increased Akt kinase activity, as measured through Akt phosphorylation at Thr638, which was effectively prevented by the intra-renal transfection of both Hsp90 isoforms. This result is consistent with a previous study demonstrating that IR injury is associated with Akt increased phosphorylation [57]. Therefore, this kinase seems to be not involved in eNOS phosphorylation at Ser1777 during IR injury. Moreover, it has been reported that Akt phosphorylates the transcription factor FOXO3a, which induces cell cycle arrest and favors the development of fibrosis after IR injury [38, 57]. Therefore, we also evaluated the expression of this transcription factor and its activating phosphorylation at Thr32. Accordingly, we observed an increase in FOXO3a phosphorylation in un-transfected and phosphorylation at Thr638, which was effectively prevented by the intra-renal transfection of Hsp90 isoforms. This result is consistent with previous studies showing that Hsp90 is involved in maintaining the stability of PKCα or Hsp90β expression or activating phosphorylation were observed among the groups studied. In contrast, a significant increase in the expression of Rho kinase was observed in un-transfected or EV-transfected IR rats, consistent with the increased eNOS phosphorylation at Thr495 in the kidneys of these animals. Accordingly, it has been demonstrated that Rho kinase activation mediates the reduction of renal blood flow in IR rats [59]. In contrast, this increment was not observed in the IR groups previously transfected with the Hsp90 isoforms.

These data together suggest that development of new therapeutically drugs able to induce Hsp90α or Hsp90β expression might be a useful to prevent the renal injury induced by IR in patients which are known to be at high risk of developing AKI, such as patients undergoing cardiac surgery or kidney recipients from deceased donors.

In summary, we showed that eNOS–Hsp90 coupling is affected during IR injury, potentially reflecting the demand for Hsp90 in other processes to recover cellular homeostasis and viability, which could be reversed through the overexpression of Hsp90. This study is the first to our knowledge to demonstrate that the intra-renal transfection of Hsp90α or Hsp90β conferred similar protection against IR injury.

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